

In vitro and *in vivo* peppermint (*Mentha piperita*) growth promotion by nonmycorrhizal fungal colonization

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Summary

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- Here peppermint growth and terpene production of *in vitro* generated plants (*Mentha piperita*) in response to inoculation with a leaf fungal endophyte were characterized.
- Peppermint plants were studied by means of morphometric, biochemical and image analysis, employing both *in vitro* and in pot cultures. Leaf essential oils were analysed by gaschromatography-mass spectrometry.
- The endophyte induced profound effects on the growth of peppermint, which responded with taller plants bearing more expanded leaves. The observed increase of leaf dry matter over leaf area suggested a real improvement of peppermint metabolic and photosynthetic apparatus. Root architecture was of the herring-bone type, showing greater dry biomass percentage over the total. A sustained lowering of (+)-menthofuran and an increase of (+)-menthol percentage concentrations were found in plants from both *in vitro* and pot cultures.
- The study represents the first report on specialized endophytic fungi in peppermint green tissues and highlights some of the principal morphological and biochemical aspects of this mutualism. Effects exerted on plant growth and essential oil production in peppermint suggest further biotechnological applications.

Key words: peppermint, leaf endophytes, fungi, PGP-HSF *Mentha* isolate, growth promotion, secondary metabolism, essential oils, *in vitro* culture.

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Introduction

Mutualism has been the prevailing concept under which the evolution and ecology of endophytes have been interpreted. Protection from invasive and harmful fungi was probably the first benefit provided by endophytic fungi to their host plant (Brundrett, 2002). Paleobotanical and morphological evidences suggested that fungal endophytes inhabiting primitive land plants might have largely been restricted to intercellular spaces within host tissues. Evolutionary changes occurred at the membrane and cell wall level of plants and fungus resulting in the specialized interface structures typical of modern mycorrhizal associations (Brundrett, 2002).

Mycorrhizal fungi, a heterogeneous group of soil fungi that colonizes the roots of most terrestrial plant species, are well known owing to the beneficial effects – better growth and higher ecological fitness – they exert on host plants (Scannerini *et al.*, 2001). These associations are generally true

mutualistic symbioses, resulting from plant and fungal host coevolution.

By contrast, with nonmycorrhizal fungi that colonize symptomlessly the living internal tissues of their host plant for all or nearly all their life cycle (endophytic fungi or endophytes, in keeping with Petrini, 1991), mutualism cannot be considered a feature common to all species. Most endophytic associations are characterized by low fungus efficiency at mineral nutrient acquisition from soil, null or scant specialisation of interface hyphae, owing to their restriction to host intercellular spaces and by low energy efficiency in plant–fungus metabolite exchange (Brundrett, 2002).

Nevertheless, literature on systemic colonization of perennial plants by endophytic fungi is increasing and has shown the existence of plant–endophyte associations with similar or higher degrees of complexity with respect to mycorrhizae (Glenn *et al.*, 1996; Moy *et al.*, 2000; Jumpponen, 2001; Redman *et al.*, 2001).

The effects of endophytes on their host plants are even more differentiated. They can act as defenders against predators (Siegel & Bush, 1996), growth promoters (Bacon & White, 2000) and competitors of microbial pathogens (Scannerini *et al.*, 2001). Temperature and/or drought tolerance acquisition by plant host due to fungal colonization have been also described (Hill *et al.*, 1991, 1996).

The vegetative growth enhancement shown by many grass species in the presence of their fungal symbionts has been principally attributed to increased plant fitness (Belesky & Fedders, 1996; Hill *et al.*, 1996; Belesky & Malinowski, 2000). Therefore in many leaf and wood endophytes, it is not always easy to determine when plant growth enhancement is a true morphogenetic response to fungal colonization. This happens when the mutualistic fungus produces chemical defences towards host pathogens (Gary *et al.*, 1997 and references therein; Cordier *et al.*, 1998) and herbivores (Richardson, 2000) or when it is able to confer drought, soil acidity, and mineral stress resistances in the endophyte-infected grass (Belesky & Malinowski, 2000).

Endophytic fungi therefore represent an interesting experimental model for studies concerning biochemical and genetic mechanisms supporting mutualism in plant–fungus interactions. Besides, they may be cultivated *in vitro* and applied to plant hosts under controlled experimental conditions in order to analyse their potential on plant morphogenesis and secondary metabolism. Varma *et al.* (2001) have recently proposed the biotechnological application of *Piriformospora indica*, a culturable mycelium possessing growth promoting effects in a vast range of plant hosts (Varma *et al.*, 2001).

A hyaline sterile fungus forming epiphyllous mycelial nets was isolated from meristem cultures of *M. piperita* (Mucciarelli *et al.*, 2002). Histological studies indicated that the culture isolate is able to colonize stems and leaves with no damage to the host plant (Mucciarelli *et al.*, 2002). *In vitro* grown peppermint plants displayed enhanced vegetative growth when infected by the fungus, with mycelium extending from green tissues to growing rootlets. The production of very thin hyphae growing away from host meristems and the asymptomatic nature of the symbiosis were commonly observed in cultures, where the isolate never sporulated. No attribution to a precise morphospecies was therefore possible and the fungal isolate was named PGP-HSF (plant growth promoter-hyaline sterile fungus). Through comparison of the 18 S rDNA sequence with those available in literature and in GenBank it was possible to determine that the mutualist of peppermint is a member of the Pyrenomycetes, belonging to the subclass Sordariomycetidae (Mucciarelli *et al.*, 2002).

The objective of the present study was to measure the morphological and biochemical responses of peppermint to PGP-HSF inoculation in order to highlight some of the principal aspects of this mutualism and its regulation. Plant responses were tested, employing two different culture systems: tissue culture and in-pot plant inoculation. Experimental trials were

performed by simple application of fungal filtrates. This was to enable identification of culture conditions most useful to the growth of the two partners and future biotechnological applications of the endophytic mycelium.

Materials and Methods

Plant material

Cuttings of *Mentha piperita* L. were cultivated in experimental plots of the Botanical Garden of Turin as described earlier (Maffei *et al.*, 1989), and transferred to an environmentally controlled chamber (26/22°C day/night with 16-h photoperiod, 144 $\mu\text{mol m}^{-2} \text{s}^{-1}$ Fluorescent lamps, 60–70% rh). 60-d-old-plants grown in pot were used as source material for both *in vitro* and *in vivo* cultures.

In vitro cultures

Micropropagated plants were obtained from 3-wk-old axillary buds developed on axenic stem cuttings as previously described (Mucciarelli *et al.*, 2002) and used as plant source for *in vitro* and in-pot experiments. Cultures were conducted on MS medium (Murashige & Skoog, 1962) supplemented with 2% sucrose, 1 mg l⁻¹ thiamine and 1% (w/v) agar in Sigmaware™ culture tubes (25 × 150 mm) provided with cotton plugs. Procedures were conducted aseptically. All *in vitro* cultures were carried out in a growth chamber (26/22°C day/night with a photoperiod of 16 h, 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Fungal solid culture

The fungal endophyte PGP-HSF (GenBank accession number AF292054) was isolated from *Mentha piperita* explants as previously described (Mucciarelli *et al.*, 2002). PGP-HSF mycelium was routinely kept on malt extract agar (MEA) slants at 25°C (MUT 88) where it does not sporulate. Inoculation of micropropagated plants was achieved starting from fungal cultures transferred on MS medium and grown for 30 d under the same conditions of plants. Colony morphology and growth of the isolate were routinely checked for contamination, and mycelium identity confirmation.

Mycelium liquid culture and bioreactor production

PGP-HSF liquid cultures were started in 500 ml-flasks containing 200 ml of autoclaved ME liquid medium and inoculated with three mycelial disks cut from 60-d-old MEA cultures on solid media. Flask cultures were kept on a giratory shaker (120 rpm) and incubated for 15 d at 25°C till a dense mycelial suspension was generated. Three-day-old subcultures of the latter were subsequently employed to start bioreactor cultures. A stirred tank bioreactor Chemap 3000 equipped with a 7-l vessel was employed. After autoclaving the bioreactor culture

vessel at 121°C for 20 min, 200 ml of mycelial suspension (0.6 g mycelial f. wt) were injected in 5 l of sterile ME medium kept at 29°C and supplemented with an antifoaming solution (Silicon Antifoam, Fluka, 1 ml l⁻¹). Culture medium was aerated at 3–5 l min⁻¹ with an impeller rate of 150 rpm and pO₂ value kept at 50%. At 2-d intervals, 50 ml of culture broth were removed aseptically under pressure in order to measure fungal biomass growth, check viability and exclude contamination. Depending on the size of the starting inoculum, 5–7-d-old-cultures, were usually harvested before reaching the stationary phase and prepared for *in vivo* inocula. Mycelial suspension, removed from the vessel, washed and collected through centrifugation was kept at 4°C. Mycelial suspension was inoculated onto plants by suspending in fresh medium.

In vitro plant inoculation

Thirty-day-old micropropagated plants were employed as the source of apexes to be used in experiments for *in vitro* plant inoculations. Young shoots, excised behind the second pair of leaves from the top were transferred to Sigmaware™ culture tubes (25 × 150 mm) provided with cotton plugs and containing the same MS medium used for peppermint micropropagation. Culture tubes destined for plant-fungus coculture were each first inoculated with a 4-mm mycelial disk, placed at the centre of the agar surface; immediately afterwards, peppermint shoots were carefully excised and inserted inside the fungal disk. For each experiment 30 inoculated (PGP-HSF+) and 30 noninoculated shoots (PGP-HSF-) were processed and each experiment run in triplicate.

Ex vitro plant inoculation

Plant microcuttings obtained from *in vitro* cultures, as described before, were rooted on a slant of moistened sand. Twenty-day-old microcuttings up to 6-cm long were planted 3 cm deep at the centre of black plastic pots (1.5-l) filled with heat sterilized (150°C overnight) quartz sand. For plants to be inoculated, roots of cuttings were partially immersed into 10 ml of liquid mycelium and subsequently placed into the sand previously syringed with other 10 ml of mycelium in order to allow contact with the fungus. Cuttings were grown in a growth chamber at 26/22°C day/night with the same photoperiod of the *in vitro* cultures.

For all experiments 20 inoculated cuttings (PGP-HSF+) and 20 noninoculated cuttings (PGP-HSF-) were processed and each experiment run in triplicate. Plants were watered every 2 d with Long Ashton solution (Hewitt, 1966).

Morphometric analyses

Micropropagated plantlets and plants grown in pots were analysed after 30 d, necessary to a complete plant development *in vitro* and for blossom initiation in pot culture.

Dry weights of leaves, stems, roots and whole plants were measured after drying plant material in a oven at 105°C to a constant weight. Stem and internode length and diameter, node number per stem and plant height were measured for each plant and the corresponding root : shoot ratio (R : S) was calculated.

Computerized image analysis of leaves was run by a Optilab Image Analyzer software (Alliance Vision, Montélimar, France) on a G3 Power PC (Macintosh) as previously described (Maffei *et al.*, 1994). This allowed calculation of the following morphometric indexes: mean leaf area (LA), mean leaf perimeter (LP), leaf surface area and perimeter ratio (RH), major and minor leaf axes, and their ratio (RA), and the leaf area index (LAI), which corresponds to the ratio between the total leaf surface and the soil surface covered by the plant.

Trichome counting

Peppermint essential oil is produced by two kinds of leaf glandular structures: peltate and capitate trichomes evenly distributed on upper and lower leaf surfaces. Peltate trichomes are larger in size and are responsible for most of the peppermint essential oil production (Maffei *et al.*, 1989).

Peltate trichomes were counted directly on both sides of each leaf pair from at least five plants, by means of a stereoscopic microscope at a magnification of 40× (Zeiss, Oberkochen, Germany). Trichome number per leaf pair was determined. Trichome density was expressed as the ratio between mean trichome number per leaf pair and the corresponding leaf area.

Biochemical parameters

Plant proteins were extracted by an IKA Ultraturrax homogenator in 50 mM Bicine (N,N-Bis [2-hydroxyethyl]-glycine) (Sigma-Aldrich, Milano, Italy) buffer, pH 7.8 and evaluated following the method of Bradford (1976) using BSA (bovine serum albumine) as a standard.

Total chlorophylls were extracted in acetone 80% aq. and analysed spectrophotometrically according to MacKinney (1941) at 663 nm with a Pharmacia Ultraspec 3000 spectrophotometer (Amersham, Milano, Italy).

Total phenols were isolated from peppermint leaves, stems and roots by plant homogenisation in a IKA Ultraturrax, solvent extracted with ethanol 70% aq. and quantified with the Folin-Ciocalteu reagent (Sigma-Aldrich) according to Bray & Thorpe (1954). Three replicates were run for each assay.

Essential oil analysis

According to the type of culture and plant size, 1–10 g of freshly picked leaves were distilled from at least three plants in a Likens and Nickerson apparatus as described in detail elsewhere (Maffei & Chialva, 1990). Gas chromatographic analyses were performed with a Hewlett-Packard 6890 gas

Table 1 Summary of growth and morphometric parameters of endophyte-inoculated (PGP-HSF+) and endophyte-free (PGP-HSF-) *Mentha piperita* plants grown *in vitro* for 30-d

Variable	PGP-HSF- Mean \pm SD	PGP-HSF+ Mean \pm SD	Mean differences	PH-test
Plant height (cm)	5.63 \pm 0.85	9.67 \pm 0.5	+4.04	*
N° of nodes	7.83 \pm 0.26	9.83 \pm 0.60	+2.00	*
Internode length (cm)	0.73 \pm 0.09	0.99 \pm 0.06	+0.26	ns
Plant f. wt (mg)	125.10 \pm 0.01	500.70 \pm 0.04	+375.6	**
Plant d. wt (d./f. wt%)	8.70 \pm 0.001 (6.95)	29.20 \pm 0.003 (5.83)	+20.5	** (**)
Leaves f. wt (mg)	95.40 \pm 0.01	269.30 \pm 0.02	+173.9	**
Leaves d. wt (d./f. wt%)	5.90 \pm 0.001 (6.18)	21.30 \pm 0.001 (7.91)	+15.4	** (**)
Stem f. wt (mg)	29.70 \pm 0.003	231.40 \pm 0.030	+201.7	**
Stem d. wt (d./f. wt%)	2.40 \pm 0.000 (8.08)	11.50 \pm 0.002 (4.97)	+99.1	** (**)
Root f. wt (mg)	24.20 \pm 0.002	422.40 \pm 0.06	+398.2	**
Root d. wt (d./f. wt%)	0.70 \pm 0.00 (2.89)	13.90 \pm 0.002 (3.29)	+13.2	** (**)
R : S	0.21 \pm 0.021	0.83 \pm 0.090	+0.62	**
LAI	0.95 \pm 0.10	2.60 \pm 0.27	+1.65	**
Leaf area (cm ²)	0.36 \pm 0.02	0.66 \pm 0.04	+0.30	**
Leaf perimeter (cm)	2.40 \pm 0.07	3.28 \pm 0.11	+0.88	**
RH	0.14 \pm 0.01	0.19 \pm 0.01	+0.05	**
Major axes (cm)	1.03 \pm 0.04	1.43 \pm 0.05	+1.40	**
Minor axes (cm)	0.43 \pm 0.01	0.55 \pm 0.01	+0.12	**
RA	2.47 \pm 0.09	2.60 \pm 0.09	+0.13	ns

d./f. wt percentage, percentage of d. wt on total weight; R : S, root–shoot ratio; LAI, leaf area index; RH, hydraulic ratio; RA, major-minor axes ratio. PH-test, Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns not significant. At least three replicates were performed for each treatment.

chromatograph, equipped with a HP 3396 integrator, employing a 50-m cross-link fused silica column HP-FFAP (Hewlett-Packard, Milano, Italy). Analyses were run under the following conditions: injection temperature 250°C, flame ion detector at 260°C, helium carrier gas flow: 1 ml min⁻¹. Temperature programming was as follows: 6 min isothermal at 60°C, then a linear temperature rise of 1.5°C min⁻¹ to 160°C, followed by an isothermal of 2 min and then a linear temperature rise of 3°C to 200°C, final isothermal of 20 min. Peak identification was based on both retention time comparison with pure standards and GC/MS as described earlier (Maffei *et al.*, 1999).

Statistics

Data were statistically processed using a standard Anova. Significant differences between treatments were determined using the Post Hoc test with Tukey-Kramer HSD simultaneous pairwise main comparison.

Results

In vitro cultures

In micropropagated peppermints, plant growth was significantly affected by PGP-HSF inoculation after 30-d of culture (Fig. 1). Effects on plant growth were evident 9–10 d after incubation and by the end of culture (day 30) plant height registered a 72% average increase in the inoculated plants in comparison with the controls. The increase resulted

from the higher mean number of stem nodes and leaf pairs per plant, as well as increases in total plant f. and d. wts (differences in single node elongation were not significant; Table 1).

Green biomass increments registered for the PGP-HSF+ plants in comparison with the controls (+182% and +679% increases of leaf and stem f. wts, respectively; Table 1) corresponded to a sustained enhancement of leaf d. wt (+28.0% and +78.1% increases of total and percentage d. wt, respectively) and on to an extensive hydration of stem tissues (–38.5% percentage d. wt; Table 1).

The development of the roots reflected stimulation by endophytic colonization. In contrast with the control plants, PGP-HSF-inoculation led to a sustained extension of the root system of micropropagated plants (Table 1). PGP-HSF-plants showed greater total f. and d. wts of roots and an increase of the root d. wt percentage (+13.8%; Table 1). As shown in Fig. 1, in PGP-HSF+ plants the intense lateral branching induced by the endophyte resulted in a well-developed herring-bone root system.

The strong stimulation of peppermint root system extension by the endophyte almost paralleled the growth promotion of the above ground plant organs as witnessed by an average R : S ratio of 0.83 in PGP-HSF+ plants.

A 174% increase of leaf area index (LAI) characterized PGP-HSF+ micropropagated plants and depended not only on the larger number of leaf pairs per plant but also on the larger average leaf surface (+83%) (Table 1). The latter was the result of increased leaf dimensions as documented

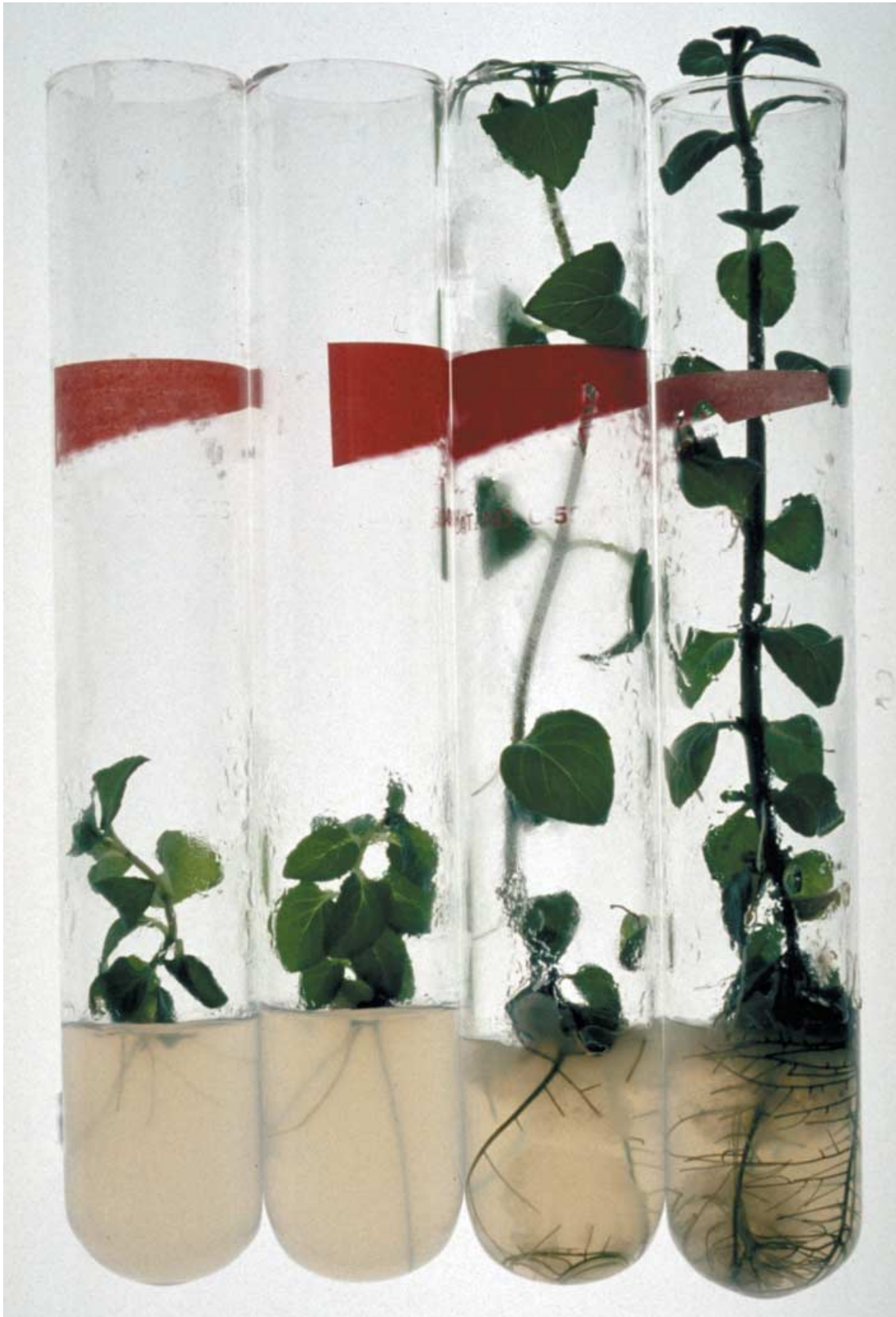


Fig. 1 Micropropagated peppermint (*Mentha piperita*) plants at the end of the incubation period. The growth response induced by the endophyte is clearly depicted by the two PGP-HSF+ plants (on the right) having the same age of the controls (two tubes on the left).

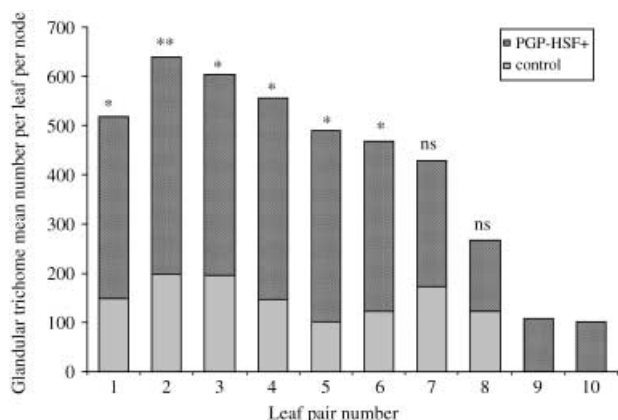


Fig. 2 Mean numbers of trichomes per leaf given for each leaf pair in PGP-HSF+ (dark columns) and PGP-HSF- (shaded columns) 30-d micropropagated peppermint (*Mentha piperita*) plants. Differences in trichome numbers within treatments were significant starting from the first apical node (leaf pair one) up to the sixth leaf pair (* symbols indicate significant differences between means according to variance analysis and Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns, not significant).

by average perimeter and number of major and minor leaf axes of PGP-HSF+ plants. On the other hand, no variation of the overall shape of leaf lamina was induced, since the main leaf axes ratio (RA) remained substantially unvaried, while the ratio between leaf area and perimeter (RH) changed proportionally to the leaf perimeter increase (Table 1).

Fig. 2 depicts the total average number of trichomes per leaf pair in PGP-HSF+ and PGP-HSF- micropropagated peppermint plants. In PGP-HSF+ plantlets, where two additional leaf pairs were normally present (nodes 9 and 10), a greater glandular trichome number per leaf with respect to PGP-HSF- plants was recorded (Fig. 2). Differences in trichome numbers were significant starting from the first apical node up to the sixth leaf pair. Differences of trichome number for leaf pairs seven and eight were not statistically significant (Fig. 2), possibly owing to their pre-existence on peppermint cuttings at the moment of apex inoculation. No significant

differences were present between PGP-HSF- and PGP-HSF+ leaf trichome densities (data not shown).

Table 2 reports the biochemical data concerning total soluble proteins, chlorophylls and phenols in 30-d-old micropropagated plants. As shown, no significant variation in soluble proteins and in both total and a : b chlorophyll ratio were observed when data were expressed as mg g^{-1} of leaf f. wt. On the contrary, PGP-HSF+ plants showed a 343% increased amount of total phenols in leaves, whereas stem and root phenol contents did not vary significantly between treatments.

Essential oil gas-chromatographic analyses showed some important differences between PGP-HSF+ and PGP-HSF- plants (Table 3). These principally consisted of changes of relative percentages of the peppermint main compounds, and to a lesser extent of significant variation of essential oil yield. Among the main components of the essential oil, the relative percentages and yield ($\mu\text{g g}^{-1}$ f. wt) of (-)-limonene and (-)-menthone did not differ significantly between variants, while the differences between (+)-menthofuran percentages were highly significant, with a 32% decrease in PGP-HSF+ plants. Even greater and highly significant was the decrease of percentage and yield of menthyl acetate (99.5% and 90.5% less in PGP-HSF+ essential oil, respectively). In contrast (-)-menthol increased in peppermint essential oil by 65% of percentage concentration in PGP-HSF+ plants in comparison with the controls.

Pot cultures

Growth promotion by PGP-HSF inoculation was evident in pot cultures as well. PGP-HSF+ plants after 30-d incubation appeared more vigorous than PGP-HSF- plants, with larger leaves, thicker stems and numerous axillary buds and stolons than normally present in control plants (Fig. 3). This response corresponded to an average 127% increase of total green f. wt of PGP-HSF+ plants. Fresh weight increase of green biomass (+108% and +137% of leaves and stems, respectively; Table 4) in PGP-HSF+ plants was accompanied by a significant proportional increase of dry biomass, thus resulting in no

Table 2 Biochemical features of endophyte-inoculated (PGP-HSF+) and endophyte-free (PGP-HSF-) *Mentha piperita* plants grown *in vitro* for 30-d

Variable	PGP-HSF- Mean \pm SD	PGP-HSF+ Mean \pm SD	Mean differences	PH-test
Total chlorophyll (mg g^{-1} f. wt)	3.21 \pm 0.85	2.70 \pm 0.22	-0.51	ns
Chlorophyll a	2.28 \pm 0.61	1.94 \pm 0.15	-0.34	ns
Chlorophyll b	0.93 \pm 0.24	0.76 \pm 0.07	-0.17	ns
Chlf. a/b ratio	2.47 \pm 0.03	2.57 \pm 0.03	-0.10	ns
Soluble proteins (mg g^{-1} f. wt)	12.20 \pm 2.46	7.27 \pm 1.43	-4.93	ns
Leaf phenols (mg g^{-1} f. wt)	1.56 \pm 0.12	6.91 \pm 0.17	+5.35	**
Stem phenols (mg g^{-1} f. wt)	1.62 \pm 0.31	4.04 \pm 1.26	+2.42	ns
Root phenols (mg g^{-1} f. wt)	3.55 \pm 0.55	2.94 \pm 1.19	-0.61	ns

PH-test, Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns, not significant. At least three replicates were performed for each treatment.

Table 3 Essential oil composition (Mean percentage) and concentration ($\mu\text{g g}^{-1}$ leaf f. wt) of endophyte-inoculated (PGP-HSF+) and endophyte-free (PGP-HSF-) *Mentha piperita* plants grown *in vitro* for 30-d

Components	PGP-HSF- Mean % \pm SD	Mean $\mu\text{g g}^{-1}$ \pm SD	PGP-HSF+ Mean % \pm SD	Mean $\mu\text{g g}^{-1}$ \pm SD	PH- <i>t</i> %	PH- <i>t</i> $\mu\text{g g}^{-1}$
α -pinene	0.59 \pm 0.16	3.73 \pm 0.39	0.38 \pm 0.08	5.27 \pm 2.24	ns	*
β -pinene	0.71 \pm 0.11	6.02 \pm 2.27	0.52 \pm 0.14	5.17 \pm 1.88	ns	ns
sabinene	0.30 \pm 0.05	2.54 \pm 1.06	0.33 \pm 0.05	3.81 \pm 1.57	ns	ns
myrcene	0.23 \pm 0.02	2.12 \pm 0.82	0.35 \pm 0.03	4.28 \pm 1.72	ns	*
limonene	3.17 \pm 0.62	25.80 \pm 9.88	2.97 \pm 0.22	38.88 \pm 15.02	ns	ns
1,8-cineole	0.17 \pm 0.02	1.56 \pm 0.64	0.15 \pm 0.01	1.70 \pm 0.63	ns	ns
menthone	0.86 \pm 0.08	9.07 \pm 4.79	0.89 \pm 0.10	9.26 \pm 2.33	ns	ns
menthofuran	51.87 \pm 4.68	485.78 \pm 197.40	35.43 \pm 2.97	405.34 \pm 140.22	**	ns
isomenthone	1.39 \pm 0.32	17.42 \pm 7.00	2.05 \pm 0.49	31.29 \pm 13.36	ns	ns
menthyl acetate	6.10 \pm 2.21	45.08 \pm 24.86	0.48 \pm 0.10	4.28 \pm 0.73	*	*
neomenthol	0.64 \pm 0.07	7.21 \pm 3.54	0.77 \pm 0.11	10.64 \pm 3.32	ns	ns
α -terpineol	0.47 \pm 0.07	5.59 \pm 2.43	0.81 \pm 0.02	9.83 \pm 3.27	ns	ns
β -caryophyllene	0.42 \pm 0.18	3.95 \pm 1.50	0.31 \pm 0.08	3.35 \pm 1.33	ns	ns
menthol	32.95 \pm 7.29	425.93 \pm 189.82	54.30 \pm 3.03	683.79 \pm 198.00	**	ns
germacrene D	0.19 \pm 0.10	1.48 \pm 1.08	0.16 \pm 0.06	1.21 \pm 0.51	ns	ns
piperitone	0.08 \pm 0.01	1.52 \pm 0.34	0.11 \pm 0.01	1.46 \pm 0.55	ns	ns
Total yield	100	1044.8 \pm 445.7	100	1219.6 \pm 383.0	ns	ns

PH-*t*, Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns, not significant. At least three replicates were performed for each treatment.

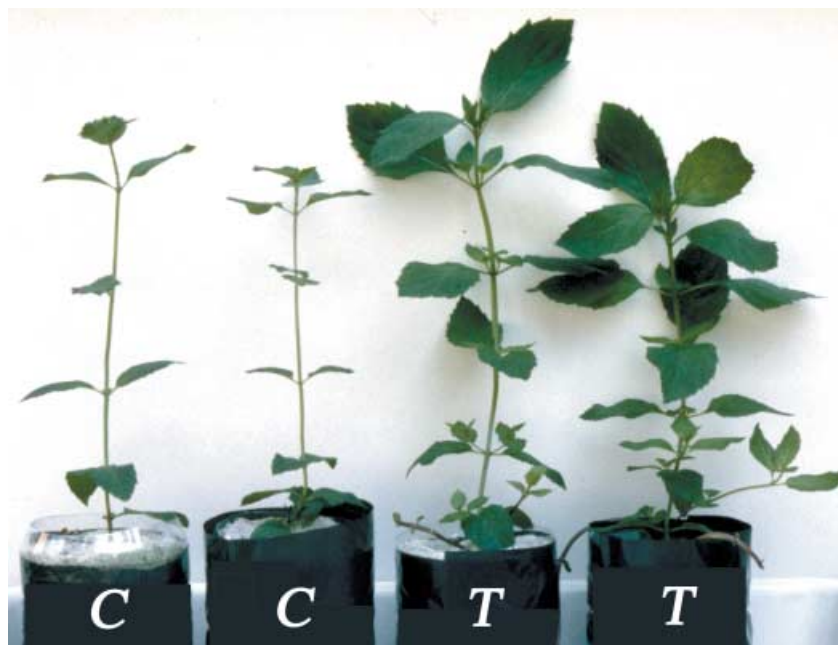


Fig. 3 Peppermint (*Mentha piperita*) plants after 30 d of culture in pots. The two plants on the right have been grown in the presence of PGP-HSF mycelium (T). Note the enlargement of leaf areas, the thickening and lateral branching of stems in PGP-HSF+ plants.

significant differences in percentages of d. wt between PGP-HSF+ and PGP-HSF- plants (Table 4).

With respect to *in vitro* grown plantlets, no differences of plant total height, number of nodes and node length were registered between PGP-HSF+ and PGP-HSF- plants (compare Table 2 and Table 4).

In contrast to the *in vitro* cultures, the roots of peppermint plants grown in pots developed normally, reaching the bottom of the pot by the end of culture (30 d) in both PGP-HSF- and PGP-HSF+ plants. PGP-HSF+ plant root biomass showed an average 100% increase in f. wt while root d. wt. did not differ significantly (Table 4). As a result, a

Table 4 Summary of growth and morphometric parameters of endophyte-inoculated (PGP-HSF+) and endophyte-free (PGP-HSF-) *Mentha piperita* plants grown in pots

Variable	PGP-HSF- Mean \pm SD	PGP-HSF+ Mean \pm SD	Mean differences	PH-test
Plant height (cm)	25.6 \pm 2.86	28.45 \pm 1.59	+2.85	ns
N° of nodes	7.20 \pm 0.20	8.17 \pm 0.48	+0.97	ns
Internode length (cm)	3.82 \pm 0.42	3.84 \pm 0.41	+0.02	ns
Plant f. wt (g)	2.68 \pm 0.34	6.98 \pm 0.35	+4.30	**
Plant d. wt (d./f. wt%)	0.38 \pm 0.021 (14.1)	0.87 \pm 0.063 (14.3)	+0.49	** (ns)
Leaves f. wt (g)	1.43 \pm 0.10	2.98 \pm 0.18	+1.55	**
Leaves d. wt (d./f. wt%)	0.21 \pm 0.01 (14.7)	0.43 \pm 0.03 (14.4)	+0.22	** (ns)
Stem f. wt (g)	0.89 \pm 0.09	2.11 \pm 0.24	+1.22	*
Stem d. wt (d./f. wt%)	0.12 \pm 0.010 (13.0)	0.27 \pm 0.02 (13.2)	+0.15	** (ns)
Root f. wt (g)	2.23 \pm 0.33	4.48 \pm 0.34	+2.25	**
Root d. wt (d./f. wt%)	0.24 \pm 0.06 (10.8)	0.38 \pm 0.05 (8.48)	+0.14	ns (**)
R : S	0.90 \pm 0.18	0.71 \pm 0.13	-0.19	ns
LAI	1.52 \pm 0.11	2.99 \pm 0.28	+1.47	**
Leaf area (cm ²)	6.56 \pm 0.27	11.66 \pm 0.60	+5.10	**
Leaf perimeter (cm)	11.18 \pm 0.29	15.66 \pm 0.55	+4.48	**
RH	0.57 \pm 0.01	0.70 \pm 0.02	+0.13	**
Major axes (cm)	5.03 \pm 0.14	7.17 \pm 0.26	+2.14	**
Minor axes (cm)	1.61 \pm 0.03	1.95 \pm 0.05	+0.34	**
RA	3.07 \pm 0.06	3.66 \pm 0.1	+0.59	**

d./f. wt percentage = percentage of d. wt on total weight; R : S, root-shoot ratio; LAI, leaf area index; RH, hydraulic ratio; RA, major-minor axes ratio. PH-test, Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns, not significant. At least three replicates were performed for each treatment.

substantial and significant increase of tissue hydration characterized root tissues of PGP-HSF+ plants as demonstrated by its significantly lower root d. wt percentages (Table 4). As a consequence of the proportional increase of green and root total weights, R : S ratio in PGP-HSF+ plants did not significantly differ from PGP-HSF- plants (Table 4).

Similar to *in vitro* cultures, PGP-HSF+ plants were characterized by a significant increase of LAI (+96.7%), which resulted exclusively from the increased leaf surface area (+76.8%), PGP-HSF+ and PGP-HSF- plants having the same number of primary leaves (Table 4, Fig. 3). Leaf dimensions of PGP-HSF+ plants were significantly increased as documented by greater values of average leaf area, leaf perimeter, and major and minor axes length, in comparison with the PGP-HSF- plants. Moreover, PGP-HSF+ plant morphology differed, the leaves being lanceolated with more incised margins (Fig. 3), consequently, significant changes in the main leaf axes ratio (Table 4).

There were no significant variations in soluble proteins, chlorophylls and total phenolic fractions in 30-d-old potted plants (data not shown).

Fig. 4 depicts the total average number of trichomes per leaf pair in PGP-HSF+ and PGP-HSF- plants grown in pots. Sensibly higher numbers of glandular trichomes per leaf were present in PGP-HSF+ plants, and differences were highly significant for each leaf pair in comparison with the controls (Fig. 4).

Essential oil gas-chromatographic analyses showed for both treatments a chemical oil profile typical of relatively young

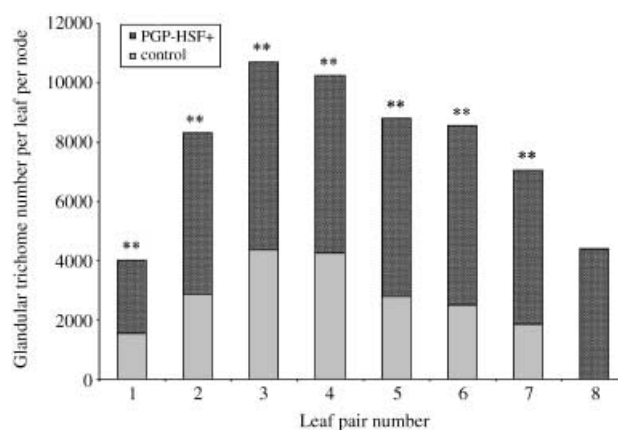


Fig. 4 Mean numbers of trichomes per leaf given for each leaf pair in PGP-HSF+ (dark columns) and PGP-HSF- (shaded columns) peppermint (*Mentha piperita*) plants grown in pots. Differences in trichome numbers within treatments were highly significant at each leaf pair (* symbols indicate significant differences between means according to variance analysis and Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns, not significant).

peppermint plants (Table 5). PGP-HSF+ and PGP-HSF- plant monoterpenes differed mostly in yield, expressed as $\mu\text{g g}^{-1}$ leaf f. wt, as in the case of the (-)-limonene (-26.3% in PGP-HSF+ plants) and for all main compounds: (-)-menthone (-17.5%) (+)-isomenthone (-16.6%) (+)-menthofuran (+30.1%), menthyl acetate (+47.8%) (+)-menthol (+40%) and (+)-neomenthol (-27.3%). Other significant variations of PGP-HSF+ plants in comparison with the

Table 5 Essential oil composition (Mean percentage) and concentration ($\mu\text{g g}^{-1}$ leaf f. wt) of endophyte-inoculated (PGP-HSF+) and endophyte-free (PGP-HSF-) *Mentha piperita* plants grown in pots

Components	PGP-HSF- Mean % \pm SD	Mean $\mu\text{g g}^{-1}$ \pm SD	PGP-HSF+ Mean % \pm SD	Mean $\mu\text{g g}^{-1}$ \pm SD	PH- <i>t</i> %	PH- <i>t</i> $\mu\text{g g}^{-1}$
α -pinene	2.58 \pm 1.48	37.97 \pm 5.95	0.67 \pm 0.11	8.40 \pm 2.45	*	*
β -pinene	1.02 \pm 0.04	11.28 \pm 1.64	1.00 \pm 0.07	10.83 \pm 3.46	ns	ns
sabinene	0.76 \pm 0.02	8.71 \pm 1.22	0.76 \pm 0.04	8.37 \pm 2.56	ns	ns
myrcene	1.81 \pm 0.31	20.80 \pm 2.77	1.86 \pm 0.38	19.69 \pm 0.48	ns	ns
α -terpinene	0.11 \pm 0.02	0.99 \pm 0.17	0.13 \pm 0.01	0.96 \pm 0.02	ns	ns
limonene	4.43 \pm 0.64	55.30 \pm 4.54	3.48 \pm 0.27	40.73 \pm 4.88	ns	*
1,8-cineole	5.85 \pm 0.46	64.20 \pm 13.20	5.77 \pm 0.58	61.57 \pm 20.35	ns	ns
γ -terpinene	1.29 \pm 0.24	12.86 \pm 2.32	1.28 \pm 0.17	12.04 \pm 0.77	ns	ns
<i>trans</i> -ocymene	0.18 \pm 0.04	1.80 \pm 0.38	0.18 \pm 0.04	1.77 \pm 0.03	ns	ns
<i>p</i> -cimene	0.29 \pm 0.06	2.95 \pm 0.57	0.28 \pm 0.05	2.45 \pm 0.21	ns	ns
menthone	29.1 \pm 2.88	338.76 \pm 10.15	28.81 \pm 2.99	279.31 \pm 12.48	ns	*
menthofuran	10.5 \pm 2.11	101.67 \pm 17.92	11.08 \pm 1.90	132.32 \pm 15.26	ns	*
isomenthone	3.00 \pm 0.22	33.34 \pm 1.40	3.05 \pm 0.44	27.79 \pm 3.41	ns	*
menthyl acetate	3.06 \pm 0.93	29.45 \pm 4.96	4.47 \pm 1.09	43.52 \pm 1.67	ns	*
neomenthol	0.80 \pm 0.10	10.12 \pm 1.16	0.63 \pm 0.10	7.38 \pm 0.98	ns	*
α -terpineol	1.77 \pm 0.18	21.98 \pm 3.48	1.33 \pm 0.21	13.06 \pm 0.87	ns	*
β -caryophyllene	4.95 \pm 0.50	61.29 \pm 11.23	4.00 \pm 0.16	45.43 \pm 4.24	*	*
menthol	18.1 \pm 4.02	208.63 \pm 34.18	21.91 \pm 4.86	292.01 \pm 28.85	ns	*
germacrene D	4.45 \pm 0.22	52.43 \pm 5.27	3.41 \pm 0.33	34.36 \pm 5.77	*	*
piperitone	6.00 \pm 1.79	76.34 \pm 4.52	4.86 \pm 1.44	49.76 \pm 6.48	*	*
Total yield	100	1150.87 \pm 178.94	100	1091.8 \pm 272.6	ns	ns

PH-*t* = Post Hoc test: * $P \leq 0.05$; ** $P \leq 0.001$; ns, not significant. At least three replicates were performed for each treatment.

controls were α -pinene (−74/−77,9% decrease in percentage and yield, respectively), α -terpineol (−40.6% in yield) and piperitone (−34.8% in yield) (Table 5), contributing to the slight decrease (−5.1%) in total productivity of PGP-HSF+ plants. Among sesquiterpenes that are typical of peppermint essential oil, β -caryophyllene and germacrene D were conspicuously and significantly decreased both in percentage concentration and in yield (Table 5) in PGP-HSF+ plants.

Discussion

The isolation from axenic leaf tissues of peppermint of a hyaline sterile endophyte able to colonize stems and leaves of to the host plant, led to the first report on the existence of plant growth promoting endophytic fungi in *M. piperita* (Mucciarelli *et al.*, 2002).

By using *in vitro* techniques it was possible to isolate in pure culture the *Mentha* endophyte and to develop an efficient culture method to obtain and maintain endophyte-free plants indefinitely. Therefore, our first approach was to test peppermint response to PGP-HSF endophyte by *in vitro* cocultures and micropropagation, finally transferring the symbiotic model to soil cultures.

PGP-HSF inoculation of peppermint plants resulted in a whole plant response. The morphogenetic effect consisted of a great increase of plant f. and d. wts, with taller plants, bearing more expanded leaves. Plants inoculated *in vitro*

resembled fully developed wild plantlets in all their growth aspects, a condition usually difficult to obtain in *M. piperita* through conventional *in vitro* culture methods.

Plant host stimulation by fungal leaf symbionts has been already documented and consisted of larger and more numerous tillers (Hill *et al.*, 1991, 1996), greater leaf elongation (Belesky & Fedders, 1996), increased leaf area, leaf thickness, stem length (Belesky & Malinowski, 2000) and occasionally altered root architecture (Malinowski *et al.*, 1999). However, most of these responses could be ascribed principally to increased plant fitness, resulting from pest-resistance or drought stress tolerance acquisition by endophyte-infected grasses (Belesky & Malinowski, 2000).

Axenic conditions, like those employed for peppermint, excluded these occurrences, thus highlighting a different role and mode of action of PGP-HSF endophyte in host growth stimulation. Moreover, *in vitro* plant inoculation with PGP-HSF resulted in a profuse colonization of the peppermint roots by the endophyte, and this could have influenced the plant response.

Jumpponen (2001) has recently observed that many plant endophytes able to colonize root tissues of land plants – dark septate endophytes (DSE) – are capable of forming mutualistic associations, functionally similar to mycorrhizas and leading to a great variety of host growth responses. Most of these effects resulted from the fact that plant root endophytes (DSE) display a biotrophic nutritional mode thanks to the presence of

interfaces which allow apoplastic and symplastic exchanges between the host and its symbiotic fungi (Jumpponen & Trappe, 1998; Jumpponen, 2001; Scannerini *et al.*, 2001).

These kind of interfaces have been described also in leaf tissues of grass endophyte associations, where the increased translocation of sugars between the symbionts has been ascribed either to changes of the photosynthetic rate of infected leaves (Bacon & White, 2000; Rodriguez Costa Pinto *et al.*, 2000) or to changes in translocation patterns of sugars within host tissues (Thrower & Lewis, 1973; White & Camp, 1995).

In peppermint, mats of anastomosed hyphae of the endophyte, with short branches apically enlarged and tightly appressed to the cuticle of leaf surfaces, were frequently observed in plant material of tissue culture derivation (Scannerini *et al.*, 2001). This situation is commonly found during leaf colonization by fungal endophytes and plant pathogens and represents a way for the fungus to enlarge the nutritional exchange surface. Considering that leaf area expansion, improvement of LAI and greater percentage dry biomass over leaf area were among the main features of PGP-HSF plant stimulation, the ability of the endophyte to exchange nutrients with peppermint leaves and eventually affect host photosynthetic efficiency deserves more consideration in future experiments, especially if considering its ability to colonize peppermint leaf apparatus.

Differently from leaves, microscopic examination of PGP-HSF inoculated roots taken from *in vitro* peppermints, showed that the peppermint endophyte formed an external enveloping mycelial sheet with no hyphal penetration into the root cortex except for a very few single senescent cells of the rhizoderm and some root hairs.

Modification of root morphology is well documented in different kinds of endophytic and mycorrhizal colonizations (Fusconi *et al.*, 1994; Berta *et al.*, 1995), and it is in AM (arbuscular mycorrhizae) that the conventional root architecture is completely changed and replaced by root dichotomic branching (Schellenbaum *et al.*, 1991), which generally gives rise to a more branched root system (Scannerini *et al.*, 2001).

PGP-HSF+ plants presented a well developed herring-bone root system and an increased root : shoot ratio. This developmental model, despite the metabolic energy cost for its maintenance allows more efficient nutritional resource exploitation by the plant and is more typical of NM (nonmycorrhizal) plants. In this regard, PGP-HSF colonization patterns differed profoundly from those of AM fungi.

Reviewing coevolution of fungi and plant roots, Brundrett (2002) has recently defined mycorrhizal fungi as dual soil-plant inhabitants which evolved to become efficient at growth and nutrient uptake in both soil and plants. On the contrary, endophytes are first of all plant inhabitants with less efficient means of acquiring nutrients from soils and which evolved to become more efficient at invading or colonizing and living within, plants (Brundrett, 2002).

However, the fact that plant endophytes do not normally interfere with host root architecture does not infer the absence of changes in root development. Preliminary results on PGP-HSF inoculated peppermint have shown increases of the total root length and of the mean diameter of the roots with respect to controls (M. Mucciarelli *et al.* unpublished), as typical of mono- and dicotyledonous AM plants (Scannerini *et al.*, 2001 and references therein). This event is generally interpreted as a way to enhance plant access to water in soil through the increase of total adsorbing area.

Various soil fungi or fungi colonizing the root surface have positive effects on host performance by the production of growth-promoting substances (Jumpponen, 2001 and references therein; Hause *et al.*, 2002). This ability has been shown also by the epibiotic fungus *Atkinsonella hypoxylon* and in a few other examples of grass endophytic associations (Clay, 1984; White & Morgan-Jones, 1996). Considering the effects on plant height, number of nodes, node length and changes in leaf shape induced *in vitro* by PGP-HSF, it is likely probable that there is hormonal stimulation in this type of cultures. *In vitro*, the mycelium spread rapidly from the apex to the culture medium, thus reaching growing rootlets. In this respect, the ability to synthesize and release hormonal substances by the fungal isolate should be assessed.

PGP-HSF+ peppermint plants grown *in vitro* showed a 343% increase in the total concentration of phenols, which was significantly different between treatments. The role of phenols in plant defence responses to pathogens (Mansfield, 1983) and endophytic colonization (Peters *et al.*, 1998) is well assessed. Schulz *et al.* (1999) showed that the accumulation of phenolic metabolites in plant tissues is a feature typical of some host–endophyte interactions and the amounts accumulated are even greater than those elicited by a fungal pathogen. This situation has been interpreted as the result of the balanced antagonism between the host and the fungus, the latter being only partially able to overcome the plant defence response (Schulz *et al.*, 1999).

Fungal-elicited accumulation of phenol-like compounds in plant hosts of clavicipitaceous endophytes has been also documented and related to host mineral nutrition improvement due to the chelating ability of phenol-like compounds (Belesky & Malinowski, 2000). Considering that in peppermint grown in pots, phenols did not accumulate, the increase of phenol-like substances observed *in vitro* is more likely to depend on the ontogenetic level of maturation reached by peppermint plants. Phenomena of polarized phenol transport towards the stem of cuttings and their accumulation in peppermint leaves could have been triggered by the intense root initiation (Torrey, 1986), which took place after fungal inoculation.

The main chemical feature of PGP-HSF+ peppermint was variation in the essential oil profile. This consisted of a significant decrease in (+)-menthofuran concentration and an almost proportional increase of (+)-menthol, both *in vitro* and in pot

cultures. Nevertheless, as a consequence of the different chemical background of the plant material at the beginning of the experiments, essential oil quality and composition were considerably different between the two culture systems as analysed at the end of the incubation period.

As for other plant secondary metabolites, regulatory mechanisms of synthetic processes leading to monoterpenes in mint have proven to be sensitive to environmental and cultural conditions (Maffei, 1988), and are influenced by plant age (Maffei *et al.*, 1989). Basically, high percentages of (+)-menthofuran are typical of young peppermint crops as a consequence of the morphogenetic level reached by secretory leaf tissue at that time. Moreover, this percentage is higher when plants experience environmental stress conditions such as unsuitable light exposure, high temperatures or pathogen attacks (Berthea *et al.*, 2001). Particularly under altered photo- and thermo-periodic cycles, the maintenance in leaf tissues of high level of oxidative conditions favours the switching on of (+)-menthofuran accumulation at the expense of (+)-menthol, through oxidation of (+)-pulegone a common precursor (Mucciarelli *et al.*, 1995; Maffei & Scannerini, 1999; Maffei *et al.*, 1999; Maffei & Scannerini, 2000). All these conditions, and especially the high level of juvenilization reached by plant tissues were satisfied in peppermint plants grown *in vitro*, where (+)-menthofuran was the main compound (51.9%) followed immediately by (+)-menthol (32%). Considering the impact shown by PGP-HSF mycelium on peppermint growth, the observed substantial modification of the essential oil was not surprising.

During *ex vitro* culture conditions, plant age was the only limiting factor to biochemical maturation of peppermint leaves. Since a more balanced proportion of (–)-menthone (29.1%), (+)-menthol (18.1%) and (+)-menthofuran (10.5%) was present from the beginning of incubation, the effect on the essential oil profile exerted by PGP-HSF through stimulation of plant morphogenesis was apparently less evident.

No significant differences were appreciable in total essential oil productivity between PGP-HSF-inoculated and control plants. From a morphogenetic point of view this correlated well with the observations that PGP-HSF inoculation did not affect glandular trichome number per unit leaf surface in peppermint plants. In *Mentha piperita*, trichome density is under the control of ontogenetic processes leading to leaf expansion and maturation, and this allows a constant number of glandular trichomes to be differently distributed on the leaf lamina of peppermint during its progressive expansion (Maffei *et al.*, 1989). A greater total number of glandular trichomes corresponded to a greater mean leaf area and LAI of PGP-HSF+ plants. This is different from the increase in plant productivity, which can result only in gene up-regulation in the metabolic apparatus of leaves or eventually in an increase of leaf trichome density (Maffei *et al.*, 1994).

A true effect on peppermint terpenoid productivity was not easy to observe and interpret in our plants. We found

significant changes in productivity of PGP-HSF+ plants only when considering the single terpenoids. In peppermint grown in pots, an increase in relative yield of (+)-menthol and (+)-menthofuran and a consequent decrease in (–)-menthone, which paralleled their relative percentages variations in the oil were documented. In peppermint, photosynthetic electron transport inhibition by the inhibitors of photosynthesis Diuron and Paraquat, caused a decline in the content of monoterpene alcohols, suggesting that the photosynthetic NADPH₂ production may be at least partially responsible for the progressive transformation of monoterpene ketones to alcohols (Maffei & Codignola, 1990). Increased rates of both carbon availability and reducing equivalent may be responsible for such a conversion (Maffei & Codignola, 1990) and this could be happening in PGP-HSF+ plants.

Other terpenoids whose concentration was affected by PGP-HSF inoculation were α -terpineol, β -caryophyllene, germacrene D and piperitone, all of great interest as synthetic precursors of other terpenoids and as potential signalling molecules with allelopathic activity (Fischer, 1991).

The endophyte of *Mentha*, PGP-HSF, has been described as growing over peppermint leaf surfaces. The individual hyphae of the epiphyllous mycelium were observed to run sparsely within peltate and capitate glandular trichomes of both upper and lower leaf surfaces, with no apparent signs of disease (Scannerini *et al.*, 2001). Glandular trichomes represent a highly efficient chemical apparatus developed by many aromatic plants to discourage bacterial and fungal colonization; hence their involvement in plant defence. Considering the well-known effects of essential oils on fungal growth and spore formation (Inouye *et al.*, 1998), the variations observed in peppermint oil profile could assume a role of relevance in a systemic endophytism like that of PGP-HSF in *Mentha piperita*.

The potential role of plant secondary metabolites in regulating mycorrhizal relationships is well recognized. As efficiently proposed by Brundrett (2002) about coevolution of roots and mycorrhizas of land plants, balanced mycorrhizae occur within plant organs that have evolved in part as specialised habitats for fungi by increasing the efficiency of and/or limiting the extent of mycorrhizal associations (Brundrett, 2002). Balanced or beneficial endophytisms like that shown by our work may similarly have evolved in plants where the control of the symbiotic association is regulated at a chemical rather than morphological level.

In some plant lineages (Brassicales and Lamiales i.e.) the acquisition of a highly diversified and evolutionary advanced secondary metabolism (alkaloids, glucosinolates, terpenoids having defence role) could have discouraged the evolution of mycorrhizal associations, where root–hypae interfaces are essential (Brundrett, 2002). In turn, plant secondary compounds have favoured the selection within natural endophytic communities of those fungal species that are not only able to survive these defence compounds, but gain ecological advantages from their presence in host tissues.

The chemical control of fungal growth within host tissues has therefore played a key role in the determination of the nature of plant-fungus relationships (Schulz *et al.*, 2002), representing another example in this regard of the complexity of plant-fungus specialisation.

Further studies are under way to determine the bio-molecular rationale for the morphological and biochemical effects reported in this work.

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